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Neutrophil Elastase in Patients With Homozygous β-Thalassemia and Pseudoxanthoma Elasticum-Like Syndrome

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In this study we investigated the possible role of neutrophil (PMN) elastase and its natural inhibitor, α1-proteinase inhibitor (α1-PI) in the pathogenesis of the pseudoxanthoma elasticum (PXE)-like syndrome which is found in patients with homozygous β-thalassemia. We studied 30 β-thalassemia homozygotes with the PXE-like syndrome [PXE(+) group], 20 β-thalassemia homozygotes without this syndrome [PXE(−) group] and 15 healthy controls. Plasma PMN elastase concentration in the PXE(+) and in the PXE(−) group was 136.4 ± 89 and 163.8 ± 126 μg/L, respectively ($P > 0.05$). In the control group, the concentration was 42.9 ± 16.8 μg/L ($P < 0.01$ for the comparison with both patients' groups). The plasma α1-PI concentration in the PXE(+) and in the PXE(−) group was 2.28 ± 0.75 and 2.6 ± 0.96 g/L, respectively ($P > 0.05$). Using logistic regression, we studied the prognostic value for PXE of the following independent variables: number of transfusions, chelation therapy, mean hemoglobin concentration, PMN elastase concentration, α1-PI concentration, chronic transaminase elevation, and positivity for anti-HCV. None of the above variables was found to have significant prognostic value for the PXE. Plasma PMN elastase concentration is elevated in all β-thalassemia homozygotes; its role in the pathogenesis of the PXE-like syndrome in β-thalassemia can not be established, but our findings suggest that neutrophils of β-thalassemia patients are activated, since PMN elastase is a marker of neutrophil activation. Am. J. Hematol. 63:63–67, 2000.

Key words: pseudoxanthoma elasticum; β-thalassemia; neutrophils; leukocyte elastase

INTRODUCTION

The introduction of intensive transfusion and iron chelation therapy has extended the life expectancy of patients with homozygous β-thalassemia [1]. As a consequence, patients manifest signs previously unknown, because of the early mortality. We recently described a pseudoxanthoma elasticum-like syndrome in patients with β-thalassemia [2]. Pseudoxanthoma elasticum is a rare hereditary connective tissue disorder affecting mainly the elastic fibers [3], and presenting with skin lesions, occular lesions, mainly in the form of angioid streaks (AS), and arterial calcifications. In patients with major or intermediate β-thalassemia we detected skin lesions and angioid streaks in 16% and 20%, respectively, these abnormalities being more frequent in older patients [2]; in another study arterial calcifications were found in 55% of β-thalassemia patients older than 30 years [4]. Additionally, PXE lesions have been reported as a contributing factor for strokes in β-thalassemia patients [5]. The pathophysiological mechanisms underlying this association are not clear. Since PXE is a disorder of the elastic fibers, and elastase is the main enzyme involved in their degradation [6], we investigated the role of PMN elastase and its natural inhibitor, α1-PI in the pathogenesis of the PXE-like syndrome in β-thalassemia patients. To explore the genetic background of the association we determined the mutations of the β-globin gene of patients with β-thalassemia and PXE-like syndrome.

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PATIENTS AND METHODS

We studied 30 unselected β-thalassemia homozygotes from various parts of Greece, with PXE-like syndrome [PXE(+) group], and 20 consecutive β-thalassemia homozygotes without the PXE-like syndrome [PXE(−) group]. All patients were followed up in the Thalassemia Units of Laikon Hospital and Agia Sofia Children's Hospital. As a control group we used 15 healthy subjects. The three groups of participants were age- and sex-matched. Pseudoxanthoma elasticum-like syndrome was diagnosed on the basis of the presence of characteristic skin lesions [7]. Patients with AS but without skin lesions were not included in the study. Patients were diagnosed and classified to the major or intermediate form of β-thalassemia on clinical grounds and by standard hematological methods [8]. All participants were Caucasians of Greek nationality and all gave informed consent to enter the study. All participants were examined for the presence of pseudoxanthoma elasticum skin lesions with special attention to the face, neck, axillary folds, lower abdomen, and thighs. Biopsies were taken from cutaneous lesions compatible with PXE [9], and all participants underwent fundoscopic examination to detect angioid streaks [10].

Blood samples were collected only when subjects were free of intercurrent illnesses or complications, and before transfusions.

Plasma PMN elastase levels were measured by a homogeneous enzyme immune assay, using a commercial kit (PMN-Elastase Imac, Diagnostica Merck, Darmstadt, Germany). Briefly, diluted patient plasma is incubated with horseradish peroxidase conjugated (Fab′) antibody fragments against the elastase–α1-proteinase inhibitor complex. The formation of antigen–antibody complexes, which occurs in the fluid phase, activates horseradish peroxidase. After peroxidase substrate is added, the activated enzyme catalyzes a chromogenic reaction. A separate sample blank with nonspecific sheep antibody fragments conjugated to horseradish peroxidase corrects for error introduced by the sample matrix.

α1-Proteinase inhibitor plasma levels were measured by single radial immunodiffusion using a commercial kit (NOR-Partigen α1-antitrypsin Behringwerke AG, Marburg, Germany).

All patients and healthy subjects had a complete blood count and white blood cell differential count done, and additionally, hemoglobin electrophoresis was performed in all the subjects in the control group to exclude any form of hemoglobinopathy. In PXE(+) patients the mutations causing β-thalassemia were detected by dot-blot hybridization of specific oligonucleotide probes to genomic DNA amplified by the polymerase chain reaction procedure. Genomic DNA isolation from peripheral blood, amplification of DNA with Taq polymerase and screening for mutations were performed as previously described [11]. The PCR amplified DNA samples were hybridized with 11 different oligonucleotide probes which detected the following mutations: IVS 1-110, -39, IVS 1-1, IVS 1-6, IVS 2-1, IVS 2-745, FSC-5, FSC-6, FSC-8, -101, and -87.

Iron chelation therapy using subcutaneous pump injection was classified as inadequate (1–2 times per week), moderate (3 times per week), or good (at least 4 times per week); a scoring scale was used, with no chelation therapy scoring 0, inadequate therapy scoring 1, moderate scoring 2, and good scoring 3. Chronic transaminase elevation was defined as an elevation more than double the upper normal limit, persisting for over 6 months. For each patient, we also calculated the mean of all serum ferritin levels available and the total number of tranfusions received.

Statistical analysis of the results was performed using the statistical software package SPSS v 6.0 (SPSS Inc., Chicago, IL).

RESULTS

The age of the patients ranged from 18 to 44 years (mean 29.3 ± 6 years), and there were 11 male and 39 female patients. Thirty-three patients had major and 17 had intermediate β-thalassemia. The clinical and laboratory profile of the two groups of patients are presented in Table I. The control group consisted of 8 male and 7 female subjects, with ages ranging from 21 to 50 years (mean 28.2 ± 7.8 years).

Fifteen patients with skin lesions compatible with PXE underwent skin biopsy. On being stained with orcein, the elastic fibers were increased and displayed clumping, abnormal arrangement, and fragmentation. The histological findings were compatible with PXE in all biopsies.

Screening for mutations of β-globin gene was performed in 15 patients from the PXE(+) group. The distribution of the thalassemic mutations in these patients is shown in Table II, and it is similar to those reported in studies of the β-thalassemia mutations in the Greek population [11,12].

Mean PMN elastase plasma concentration was 136.4 ± 89 μg/L (range 40.8–386.6 μg/L) in the PXE(+) group, 163.8 ± 126 μg/L (range 18.8–540.6 μg/L) in the PXE(−) group, and 42.9 ± 16.8 μg/L (range 12.5–70.6 μg/L) in the healthy control group. Twenty-two out of thirty (73.3%) patients in the PXE(+) group and 16/20 (80%) patients in the PXE(−) group had PMN elastase plasma concentration above the upper normal limit (76.5 μg/L, defined as the mean of healthy controls plus two standard deviations). PMN elastase concentration did not differ between PXE(+) and PXE(−) patients ($P > 0.05$) but it was significantly higher in both patients groups, in comparison to healthy controls (one-way ANOVA,

TABLE I. Clinical and Laboratory Profile of Patients

Variable	Total $n = 50$	PXE(+) $n = 30$	PXE(−) $n = 20$
Age (mean)	18–44 (29.3)	19–44 (30.6)	18–40 (27.5)
Sex (male/female)	11/39	6/24	5/15
Severity (intermediate/major)	17/33	10/20	7/13
Number of transfusions (units)	560 ± 333	549 ± 355	577 ± 309
Chelation therapy score	1.7 ± 1.1	1.8 ± 1.0	1.6 ± 1.0
Anti-HCV positive	20 (40%)	11 (27.5%)	9 (45%)
HbsAg positive	2 (4%)	2 (6.6%)	None
Chronic transaminase elevation	24 (48%)	10 (33.3%)	14 (70%)
Splenectomy	36 (72%)	21 (70%)	15 (75%)
Haemoglobin (g/dL)[a]	8.9 ± 1.2	9.0 ± 1.2	8.8 ± 1.2
Ferritin (ng/mL)[a]	2063.3 ± 1643.4	1918 ± 1276.6	3046 ± 1692.1[b]

[a]Mean of all available measurements. Hemoglobin levels are measured before transfusion.
[b]$P = 0.02$.

TABLE II. Distribution of the β-Thalassemia Mutations in PXE(+) Patients

Type of mutation	*n*	%	% reported for thal. major[a]	% reported for thal. intermedia[a]
IVS 1-110	13	43.33	42.2	30.39
β-39	6	20.0	19.26	4.91
IVS 1-1	5	16.66	16.05	3.92
IVS 1-6	1	3.33	11.46	32.35
IVS 2-1	2	6.66	2.29	3.92
IVS 2-745	–	–	1.37	5.88
FSC-5	–	–	0.45	–
FSC-6	1	3.33	2.29	1.96
FSC-8	–	–	0.45	–
−101	–	–	–	0.98
−87	–	–	2.29	4.91
Unknown	2	6.66	1.83	10.78

[a]Data from reference 11.

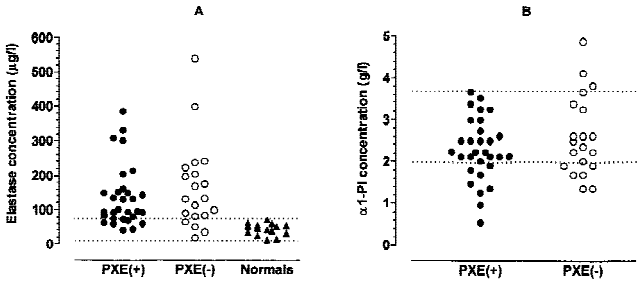

Fig. 1. Plasma concentrations of neutrophil elastase (A) and α1-proteinase inhibitor (B) in the two groups of patients. The dotted lines represent the normal range.

$P < 0.001$). The distribution of PMN elastase plasma concentration in all study groups is shown in Fig. 1A.

Mean α1-PI plasma concentration was 2.28 ± 0.75 g/L (range 0.52–3.66 g/L) in the PXE(+) group and 2.6 ± 0.96 g/L (range 1.35–4.86 g/L) in the PXE(−) group. The means of the two groups did not differ significantly (Mann-Whitney test, $P > 0.05$), and the 95% confidence intervals of both groups were within the normal range (normal range 2.0–3.66 g/L). The distribution of α1-PI plasma concentration in the two groups of patients is shown in Fig. 1B.

Neutrophil elastase concentration in β-thalassemia patients did not correlate with age, number of transfusions, chelation therapy score, mean ferritin levels, and mean hemoglobin levels (Spearman's test, $P > 0.05$ for all comparisons). There was no difference in PMN elastase concentration between splenectomized or nonsplectomized patients, between patients with or without chronically elevated levels of transaminases, and between patients positive or negative for anti-HCV (Mann-Whitney test, $P > 0.05$ for all comparisons). There were no differences in PMN elastase concentration among groups when patients were grouped according to chelation therapy score (Kruskal-Wallis test).

Using logistic regression analysis, we studied the prognostic value for PXE of the following independent variables: number of transfusions, chelation therapy score, mean hemoglobin concentration, PMN elastase concentration, α1-PI concentration, chronic transaminase elevation, and positivity for anti-HCV. None of the above variables was found to have significant prognostic value for the PXE. Ferritin levels were higher in patients without PXE-like syndrome (Mann-Whitney test, $P = 0.02$).

DISCUSSION

In the present study, we found that patients with homozygous β-thalassemia have increased plasma concentration of PMN elastase, although there was no difference in PMN elastase concentration between patients with the PXE-like syndrome and patients without this syndrome. In addition, α1-PI plasma concentration in patients with homozygous β-thalassemia, either with or without the PXE-like syndrome, did not differ significantly from nor-

mal values. Plasma PMN elastase levels are not absolutely representative of its activity at tissue level, so that although our results do not support the involvement of PMN elastase in the pathogenesis of the PXE-like syndrome, this possibility cannot be excluded.

Moreover, the fact that all patients with β-thalassemia have elevated serum levels of PMN elastase suggests that it may also have a role in the pathogenesis of other manifestations of β-thalassemia, such as cardiomyopathy or liver disease [8].

We did not find prognostic value for PXE, for any of the variables in the logistic regression analysis in the present and in a previous study. Ferritin did not have any prognostic significance for PXE in the previous study [2]. Mean serum ferritin levels were higher in the PXE(−) group in the present study, but ferritin levels in both patient groups were very high, and consequently, we could not draw conclusions from this fact.

The genotypes of β-thalassemia patients with the PXE syndrome did not differ from those of the general β-thalassemia population in Greece, therefore no association could be established between one haplotype and the appearance of the PXE syndrome. This lack of association argues against a genetic mechanism for the association of the PXE-like syndrome and β-thalassemia. Interestingly the genetic locus for autosomal PXE has been recently mapped at 16p13.1 [13,14] while the locus of the α-globin gene is mapped at 16p13.3 [15].

The presence of increased plasma concentration of PMN elastase suggests neutrophil activation in patients with β-thalassemia, since PMN elastase is released from the primary (azurophilic) granules of neutrophil leukocytes [16], during leukocyte activation [17]. The stimuli causing neutrophil activation are various (e.g., inflammation, presence of bacterial wall lipopolysachharite, and binding of immune complexes to Fc and C3 receptors), and activation is mediated through the release of mediators that act on neutrophils such as IL-8, TNF-α, IFN-γ [18].

Interleukin-8 is a mediator inducing neutrophil chemotaxis and activation expressed as a respiratory burst and PMN elastase release, so much so that PMN elastase release has been used in bioassays for IL-8 [19]. Our findings are in agreement with other studies which found increased serum levels of IL-8 in β-thalassemia patients [20,21] and increased production of IFN-γ and TNF-α by cultured blood mononuclear cells of β-thalassemia patients after stimulation with phytohaemaglutinin [22]. The above cytokines activate phagocytic cells and cause neutrophil granule content release [16]. The reasons for the increased cytokine levels, and consequently of PMN elastase levels in β-thalassemia, are not entirely understood. It has been proposed that iron overload and chronic antigenic stimulation are possible causes, but more data are needed to support this hypothesis [20–23]. Whatever the cause of neutrophil activation in β-thalassemia, it may induce tissue injury [24]. It has been suggested that PMN elastase plays a role in neutrophil transmigration, solubilizing the basement membrane and subendothelial matrices [25,26]. Neutrophils migrating to the tissues cause injury via mechanisms such as release of granule enzymes (elastase and myeloperoxidase) and free radical production [27]. Myeloperoxidase catalyzes hypochlorous acid production which inactivates α1-PI in the extravascular space, so that PMN elastase can act unopposed, degrading extracellular matrix. Oxygen free radicals produced by neutrophils are converted to the most toxic hydroxyl radical, in a reaction catalyzed by unbound iron [28]. In this study we measured plasma PMN elastase which is inactivated by α1-PI but on the other hand it reflects the extravascular PMN elastase release by neutrophils.

CONCLUSIONS

In this study we found that plasma PMN elastase levels are increased in patients with homozygous β-thalassemia, irrespective of the presence of the PXE-like syndrome, so that we could not establish its role in the pathogenesis of the PXE-like syndrome in β-thalassemia. On the other hand, our findings suggest neutrophil activation which may contribute in the pathogenesis of tissue injury in β-thalassemia.

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